

06/30/99  
jc552 U.S. PTO

A  
jc551 U.S. PTO  
09/345815  
06/30/99

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Patent Application of: **Fatih M. Uckun**  
Title: **METHOD FOR INHIBITING C-JUN EXPRESSION USING JAK-3 INHIBITORS**  
Attorney Docket No.: **957.001US1**

**PATENT APPLICATION TRANSMITTAL**

**BOX PATENT APPLICATION**  
Assistant Commissioner for Patents  
Washington, D.C. 20231

We are transmitting herewith the following attached items and information (as indicated with an "X"):


- ☒ Utility Patent Application under 37 CFR § 1.53(b) comprising:  
☒ Specification ( 33 pgs, including claims numbered 1 through 14 and a 1 page Abstract).  
☒ Formal Drawing(s) ( 5 sheets).  
☒ Unsigned Combined Declaration and Power of Attorney ( 3 pgs).  
☒ Return postcard.

The filing fee (NOT ENCLOSED) will be calculated as follows:

	No. Filed	No. Extra	Rate	Fee
TOTAL CLAIMS	14 - 20 =	0	x 18 =	\$0.00
INDEPENDENT CLAIMS	2 - 3 =	0	x 78 =	\$0.00
] MULTIPLE DEPENDENT CLAIMS PRESENTED				\$0.00
BASIC FEE				\$760.00
TOTAL				\$760.00

**THE FILING FEE WILL BE PAID UPON RECEIPT OF THE NOTICE TO FILE MISSING PARTS.**

**SCHWEGMAN, LUNDBERG, WOESSNER & KLUTH, P.A.**  
P.O. Box 2938, Minneapolis, MN 55402 (612-373-6900)

By:   
Atty: Robert J. Harris, Ph.D.  
Reg. No. 37,346

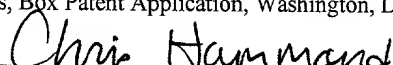
**Customer Number 21186**

"Express Mail" mailing label number: EL273421044US

Date of Deposit: June 30, 1999

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Box Patent Application, Washington, D.C. 20231.

By: Chris Hammond

Signature: 

## METHOD FOR INHIBITING C-JUN EXPRESSION USING JAK-3 INHIBITORS

### Priority of Invention

5                   This application claims priority under 35 U.S.C. §119(e) from U.S. Provisional Application Number 60/091,150, filed 30 June 1998.

### Background of the Invention

                  The protooncogene *c-jun* is the cellular counterpart of the *v-jun*  
10    oncogene of avian sarcoma virus 17. *C-jun* expression is activated in response to a diverse set of DNA-damaging agents including ara-C, UV radiation, topoisomerase II inhibitors, alkylating agents, and ionizing radiation. As an immediate early response gene that is rapidly induced by pleiotropic signals, *c-jun* may have important regulatory functions for cell cycle progression, proliferation,  
15    and survival. See Ryder, K., Lau, L. F., and Nathans, D. "A gene activated by growth factors is related to the oncogene *v-jun*," *Proc Natl Acad Sci USA*. 85: 1487-1491, **1988**; Schutte, J., Viallet, J., Nau, M., Segal, S., Fedorko, J., and Minna, J. "*jun*-B inhibits and *c-fos* stimulates the transforming and trans-activating activities of *c-jun*," *Cell*. 59: 987-997, **1989**; Neubergh, M., Adamkiewicz, J., Hunter, J. B., and  
20    Mueller, R. "A fos protein containing the Jun leucine zipper forms a homodimer which binds to the AP-1 binding site," *Nature*. 341: 589-590, **1989**; Mitchell, P. J. and Tijian, R. "Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins," *Science*. 245: 371-378, **1989**; Bohmann, D., Bos, T. J., Admon, T., Nishimura, R., Vogt, P. K., and Tijian, R. "Human protooncogene *c-jun*  
25    encodes a DNA binding protein with structural and functional properties of transcription factor AP-1," *Science*. 238: 1386-1392, **1988**; Kharbanda, S. M., Sherman, M. L., and Kufe, D. W. "Transcriptional regulation of *c-jun* gene expression by arabinofuranosylcytosine in human myeloid leukemia cells," *J Clin*

- Invest.* 86: 1517-1523, 1990; Rosette, C. and Karin, M. "Ultraviolet light and osmotic stress: activation of the JNK cascade through multiple growth factor and cytokine receptors," *Science*. 274: 1194-7, 1996; Rubin, E., Kharbanda, S., Gunji, H., and Kufe, D. "Activation of the *c-jun* protooncogene in human myeloid leukemia cells treated with etoposide," *Molecular Pharmacology*. 39: 697-701, 5 1991; Dosch, J. and Kaina, B. "Induction of *c-fos*, *c-jun*, *junB* and *junD* mRNA and AP-1 by alkylating mutagens in cells deficient and proficient for the DNA repair protein O6-methylguanine-DNA methyltransferase (MGMT) and its relationship to cell death, mutation induction and chromosomal instability," *Oncogene*. 13: 10 1927-35, 1996; Chae, H. P., Jarvis, L. J., and Uckun, F. M. "Role of tyrosine phosphorylation in radiation-induced activation of *c-jun* protooncogene in human lymphohematopoietic precursor cells," *Cancer Res.* 53: 447-51, 1993; and Karin, M., Liu, Z.-G., and Zandi, E. "AP-1 function and regulation," *Current Opinion in Cell Biology*. 9: 240-246, 1997.
- 15                    *C-jun* encodes the nuclear DNA-binding protein, JUN, that contains a leucine-zipper region involved in homo- and heterodimerization. JUN protein dimerizes with another JUN protein or the product of *c-fos* gene and forms the activating protein-1 (AP-1) transcription factor. JUN-JUN homodimers and JUN-FOS heterodimers preferentially bind to a specific heptameric consensus
- 20 sequence found in the promoter region of multiple growth regulatory genes. Alterations of *c-jun* protooncogene expression can therefore modulate the transcription of several growth-regulators affecting cell proliferation and differentiation. See Ryder, K., Lau, L. F., and Nathans, D. "A gene activated by growth factors is related to the oncogene *v-jun*," *Proc Natl Acad Sci USA*. 85: 25 1487-1491, 1988; Neubergh, M., Adamkiewicz, J., Hunter, J. B., and Mueller, R. "A fos protein containing the Jun leucine zipper forms a homodimer which binds to the AP-1 binding site," *Nature*. 341: 589-590, 1989; Karin, M., Liu, Z.-G., and Zandi, E. "AP-1 function and regulation," *Current Opinion in Cell Biology*. 9: 240-246, 1997; Angel, P., Allegretto, E. A., Okino, S. T., Hattori, K., Boyle, W. J., Hunter, 30 T., and Karin, M. "Oncogene *jun* encodes a sequence-specific trans-activator similar

to AP-1," *Nature*. 332: 166-170, **1988**; and Musti, A. M., Treier, M., and Bohmann, D. "Reduced ubiquitin-dependent degradation of c-Jun after phosphorylation by MAP kinases," *Science*. 275: 400-402, **1997**.

- C-jun* plays a pivotal role in Ras-induced transformation and has also
- 5 been implicated as a regulator of apoptosis when *de novo* protein synthesis is required. *C-jun* induction is required for ceramide-induced apoptosis and stress-induced apoptosis after UV exposure or other forms of DNA damage. This induction is thought to be triggered by activation of JUN-N-terminal kinases (JNKs) (also known as stress-activated protein kinases) which leads to enhanced *c-jun*
  - 10 transcription by phosphorylation of JUN at sites that increases its ability to activate transcription. Ectopic expression of a dominant-negative *c-jun* mutant lacking the N terminus or a dominant-negative JNK kinase abolishes stress-induced apoptosis. See Karin, M., Liu, Z.-G., and Zandi, E. "AP-1 function and regulation," *Current Opinion in Cell Biology*. 9: 240-246, **1997**; Collotta, F., Polentarutti, N., and
  - 15 Mantovani, A. "Expression and involvement of c-fos and c-jun protooncogenes in programmed cell death induced by growth factor deprivation in lymphoid cell lines," *J. Biol. Chem.* 267: 18278-18283, **1992**; Ham, J., Babij, C., Whitfield, J., Pfarr, C. M., Lallemand, D., Yaniv, M., and Rubin, L. L. "A c-Jun dominant negative mutant protects sympathetic neurons against programmed cell death,"
  - 20 *Neuron*. 14: 927-939, **1995**; Verheij, M., Bose, R., Lin, X. H., Yao, B., Jarvis, W. D., Grant, S., Birrer, K. M. J., Szabo, E., Zon, L. I., Kyriakis, J. M., Haimovitz F. A., Fuks, Z., and Kolesnick, R. N. "Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis," *Nature*. 380: 75-9, **1996**; Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. "Identification of an oncoprotein- and
  - 25 UV-responsive protein kinase that binds and potentiates the c-Jun activation domain," *Genes Dev.* 7: 2135-48, **1993**; Derijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. J. "JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain," *Cell*. 76: 1025-37, **1994**; and Chen, Y. R., Wang, X., Templeton, D., Davis, R. J.,
  - 30 and Tan, T. H. "The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by

ultraviolet C and gamma radiation. Duration of JNK activation may determine cell death and proliferation," *J Biol Chem.* 271: 31929-36, **1996**.

Protein tyrosine kinases (PTK) play important roles in the initiation and maintenance of biochemical signal transduction cascades that affect

5 proliferation and survival of B-lineage lymphoid cells. Oxidative stress has been shown to activate BTK, SYK, and Src family PTK. It is known that PTK activation precedes and mandates radiation-induced activation of *c-jun* protooncogene expression in human B-lineage lymphoid cells (Chae, H. P., Jarvis, L. J., and Uckun, F. M. *Cancer Res.* 53: 447-51, 1993). However, the identity of the PTK

10 responsible for radiation-induced *c-jun* activation is not yet known. See Uckun, F. M., Waddick, K. G., Mahajan, S., Jun, X., Takata, M., Bolen, J., and Kurosaki, T. "BTK as a mediator of radiation-induced apoptosis in DT-40 lymphoma B cells," *Science.* 273: 1096-100, **1996**; Kurosaki, T. "Molecular mechanisms in B cell antigen receptor signaling," *Curr Opin Immunol.* 9: 309-18, **1997**; Uckun F.M,

15 Evans W.E, Forsyth C.J, Waddick K.G, T-Ahlgren L., Chelstrom L.M, Burkhardt A., Bolen J., Myers D.E. "Biotherapy of B-cell precursor leukemia by targeting genistein to CD19-associated tyrosine kinases." *Science* 267:886-891, **1995**; Myers D.E., Jun X., Waddick K.G., Forsyth C., Chelstrom L.M., Gunther R.L., Tumer N.E, Bolen J., Uckun F.M. "Membrane-associated CD19-LYN complex is an

20 endogenous p53-independent and bcl-2-independent regulator of apoptosis in human B-lineage lymphoma cells." *Proc Nat'l Acad Sci USA* 92: 9575-9579, **1995**; Tuel Ahlgren, L., Jun, X., Waddick, K. G., Jin, J., Bolen, J., and Uckun, F. M. "Role of tyrosine phosphorylation in radiation-induced cell cycle-arrest of leukemic B-cell precursors at the G2-M transition checkpoint," *Leuk Lymphoma.* 20: 417-26, **1996**;

25 Qin, S., Minami, Y., Hibi, M., Kurosaki, T., and Yamamura, H. "Syk-dependent and -independent signaling cascades in B cells elicited by osmotic and oxidative stress," *J Biol Chem.* 272: 2098-103, **1997**; Saouaf, S. J., Mahajan, S., Rowley, R. B., Kut, S., Fargnoli, J., Burkhardt, A. L., Tsukada, S., Witte, O. N., and Bolen, J. B. "Temporal differences in the activation of three classes of non-transmembrane

30 protein tyrosine kinases following B cell antigen receptor surface engagement,"

- Proc Natl Acad Sci USA*. 91: 9524-28, 1994; Law, D. A., Chan, V. F. W., Datta, S. K., and DeFranco, A. L. "B-cell antigen receptor motifs have redundant signalling capabilities and bind the tyrosine kinases PTK72, Lyn and Fyn," *Curr Biol*. 3: 645-57, 1993; Hibbs, M. L., Tarlinton, D. M., Armes, J., Grail, D., Hodgson, G.,
- 5 Maglitto, R., Stacker, S. A., and Dunn, A. R. "Multiple defects in the immune system of Lyn-deficient mice, culminating in autoimmune disease," *Cell*. 83: 301-311, 1995; Aoki, Y., Isselbacher, K. J., and Pilai, S. "Bruton tyrosine kinase is tyrosine phosphorylated and activated in pre-B lymphocytes and receptor-ligated B cells," *Proc Natl Acad Sci USA*. 91: 10606-10609, 1994; Jugloff, L. S. and Jongstra
- 10 Bilen, J. "Cross-linking of the IgM receptor induces rapid translocation of IgM-associated Ig alpha, Lyn, and Syk tyrosine kinases to the membrane skeleton," *J Immunol*. 159: 1096-106, 1997; Thomis, D. S., Gurniak, C. B., Tivol, E., Sharpe, A. H., and Berg, L. J. "Defects in B lymphocyte maturation and T lymphocyte activation in mice lacking Jak 3," *Science*. 270: 794-797, 1995; Nosaka, T., Van
- 15 Deursen, J. M., Tripp, R. A., Thierfelder, W. E., Witthuhn, B. A., McMickle, A. P., Doherty, P. c., Grosveld, G. C., and Ihle, J. N. "Defective lymphoid development in mice lacking Jak 3," *Science*. 270: 800-802, 1995.

- U.S. Patent Application Serial Number 09/087,479 (entitled Quinazolines For Treating Brain Tumor; filed 28 May 1998) discloses
- 20 hydroxyquinazoline derivatives that exhibit potent cytotoxicity against human glioblastoma cells (i.e. brain tumor cells). Because JAK-3 is not known to be present in these glioblastoma cells, the cytotoxic activity of the hydroxyquinazoline derivatives is not believed to result from inhibition of JAK-3 activity. Additionally, the cytotoxic activity of the hydroxyquinazoline derivatives is not known to result
- 25 from the inhibition of c-jun activation.

There is currently a need for therapeutic agents and methods that are useful for preventing or reducing cell damage that results from exposure to radiation and chemical agents that cause DNA-damage. There is also a need for chemical agents as well as *in vitro* and *in vivo* methods that can be used to further investigate

the biological pathways associated with DNA-damage that results from exposure to radiation or chemical agents.

### Summary of the Invention

5           The invention provides a method comprising inhibiting c-jun expression in cells (e.g. mammalian or avian) by contacting the cells (*in vitro* or *in vivo*) with a substance that inhibits the activity of Janus family kinase 3 (JAK-3).

          The invention also provides a therapeutic method for preventing or treating a pathological condition in a mammal (e.g. a human) wherein c-jun  
10   activation is implicated and inhibition of its expression is desired comprising administering to a mammal in need of such therapy, an effective amount of a substance that inhibits the activity of JAK-3.

          The invention also provides novel compounds of formula I as well as processes and intermediates useful for their preparation.

15           The invention also provides substances that are effective to inhibit JAK-3 for use in medical therapy (preferably for use in treating conditions that result from exposure to radiation or to chemical agents that cause DNA damage), as well as the use of substances that inhibit JAK-3 for the manufacture of a medicament for the treatment of a condition that is associated with exposure to  
20   radiation, or to chemical agents that cause DNA damage.

### Brief Description of the Figures

**Figure 1. Radiation-induced c-jun activation in wild-type DT-40 lymphoma B-cells. [A]. *Dose response for induction of c-jun mRNA.*** DT-40  
25   chicken cells were irradiated at the indicated doses (0,10,15,20 Gy). Total RNA was extracted after a 2 hours or 4 hours post-irradiation time period. RNA (20 mg) was loaded on a Northern gel and transferred by capillary blotting to a nylon membrane. The Northern blot was hybridized with a <sup>32</sup>P labeled chicken *c-jun* probe (top panel) or a chicken GAPDH probe (bottom panel). The inset shows the values for the  
30   *c-jun*/GAPDH transcript expression ratios as determined with a Bio Rad Storage

Phosphor Imager and corresponding SI values [B]. *Effect of the PTK inhibitor genistein on induction of c-jun mRNA.* Cells were treated with 30 mg/ml of genistein for 24 hours at 37°C prior to exposure to 20 Gy ionizing radiation. *c-jun* expression levels were determined as in [A].

5 **Figure 2. Radiation-induced activation of *c-jun* in BTK<sup>-</sup> DT-40 cells.** Two representative experiments (shown in [A] and [B]) showing induction of *c-jun* mRNA expression by ionizing radiation in wild type (WT) and BTK<sup>-</sup> DT-40 cells. Poly (A)<sup>+</sup> RNA was isolated from non-irradiated cells as well as irradiated cells (20 Gy, with a 2 hours post-radiation recovery period). Northern blots of 2 mg  
10 of poly (A)<sup>+</sup> were hybridized with *c-jun* probe (*top panel*), (-actin probe (*middle panel* in [A] only), and GAPDH probe (bottom panel). The inset below each panel shows the relative expression of *c-jun* normalized for RNA load (*c-jun*/GAPDH ratio) and SI (fold induction over non-irradiated controls).

**Figure 3. Induction of *c-jun* mRNA expression by ionizing**  
15 **radiation in wild type and mutant DT-40 cell lines.** DT-40, BTK<sup>-</sup> DT-40, SYK<sup>-</sup> DT-40 (shown in [A]), as well as LYN<sup>-</sup> DT-40 and LYN<sup>-</sup> SYK<sup>-</sup> DT 40 cells (shown in [B]) were irradiated with 20 Gy and poly (A)<sup>+</sup> RNA (in [A]) or total RNA (in [B]) was harvested after a 2 hour recovery period. RNA from non-irradiated cells was used as a control. Northern blots containing 2 mg of poly (A)<sup>+</sup> (in [A]) or 20 mg of  
20 total RNA (in [B]) from each cell line were hybridized with both <sup>32</sup>P labeled *c-jun* probe (top panel) and GAPDH probe (bottom panel). The insets below the panels show the relative expression of *c-jun* normalized for RNA loading (*c-jun*/ GAPDH ratios) as well as the SI (fold induction over non-irradiated controls).

**Figure 4. JAK-3 Inhibitors. [A]. Structures of JAK-3 inhibitors.**  
25 **[B] Specificity of JAK-3 inhibitors.** Sf21 cells infected with baculovirus expression vectors for JAK-1 JAK-2 or JAK-3 were subjected to immunoprecipitation with anti-JAK antibodies. JAK-1 (shown in B.1), JAK-2 (shown in B.2) and JAK-3 (shown in B.3 and B.4 which illustrate results from 2 independent experiments) immune complexes were treated with 1% DMSO (vehicle control = CON),  
30 Compound 1, or Compound 2 for 1 hour prior to hot kinase assays, as described



(20,22). Both compounds inhibited JAK-3 when used at 10 µg/ml whereas they did not inhibit JAK-1 or JAK-2 even at 75 µg/ml [C]. EMSAs of 32Dc22-IL-2Rβ cells. Compound 1 (100 (g/ml) and Compound 2 (100 (g/ml) inhibited IL-2 triggered JAK-3-dependent STAT activation but not IL-3-triggered JAK-1/JAK-2-dependent  
5 STAT activation in 32Dc11-IL-2Rβ cells.

**Figure 5. Effects of a JAK-3 inhibitor on *c-jun* induction in irradiated DT-40 cells.** Cells were treated with the quinazoline derivative 4-(3'-Bromo-4'-hydroxyl-phenyl)-amino-6,7-dimethoxyquinazoline (100 mg/ml) for 24 hours at 37 °C prior to exposure to 20 Gy ionizing radiation. *c-jun*  
10 expression levels were determined as outlined in Figures 1-3.

#### Detailed Description

As used herein, the term “inhibit” means to reduce by a measurable amount, or prevent entirely; and the phrase “inhibit *c-jun* activation” includes the  
15 inhibition of RNA production and the inhibition of the production of the protein encoded by the RNA.

Applicants examined the potential involvement of BTK, SYK and LYN in radiation-induced *c-jun* activation, using DT-40 chicken lymphoma B-cell clones rendered deficient for these specific PTK by targeted gene disruption. It was  
20 found that BTK plays no role in radiation-induced *c-jun* activation. Similarly, neither LYN nor SYK are required for activation of *c-jun* after radiation exposure. However, their participation may influence the magnitude of the *c-jun* response. It was unexpectedly discovered, however, that an inhibitor of Janus family kinase 3 (JAK-3) abrogated radiation-induced *c-jun* activation.

25 C-jun expression can be activated by exposure to chemical agents that damage DNA such as ara-C, a topoisomerase II inhibitors, or alkylating agents. C-jun activation can also result from exposure to ultraviolet radiation or ionizing radiation. According to the invention, inhibitors of JAK-3 can be used to inhibit *c-jun* expression resulting from exposure to radiation or exposure to chemical agents.

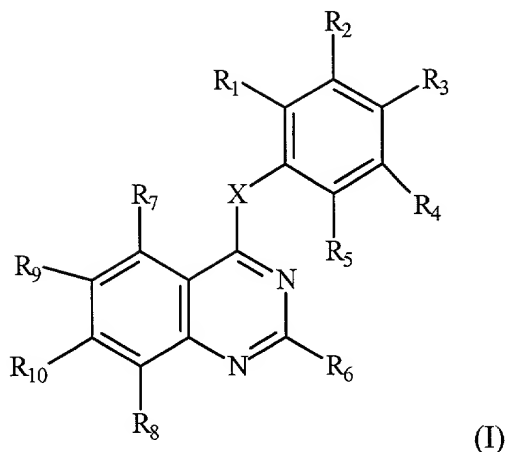
The methods of the invention can be carried out *in vitro*. Such *in vitro* methods are also useful for studying the biological processes associated with cell response to DNA damaging agents. The methods of the invention can also be carried out *in vivo*. Such methods can also be used to study the biological processes associated with cell response to DNA damaging agents, as well as for treating pathological conditions in mammals (e.g. humans) that result from exposure to DNA-damaging agents.

Pathological conditions that result from exposure to DNA-damaging agents include conditions that result from oxidative stress, such as tissue or organ (e.g. heart, liver, or kidney) damage, inflammation, and hair loss, as well as the negative effects that are produced by oxygen free radicals during chemotherapy. Oxidative stress may result from exposure to external agents, or may result from internal processes. Thus, JAK-3 inhibitors are also useful for treating conditions resulting from the action of internally generated oxygen free radicals, such as aging and amyelotrophic lateral sclerosis (ALS).

According to the invention, the JAK-3 inhibitors may be administered prophylactically, i.e. prior to exposure to the DNA-damaging agent, or the JAK-3 inhibitors may be administered after exposure to the DNA damaging agent.

The JAK-3 inhibitors useful in the methods of the invention include all compounds capable of inhibiting the activity of JAK-3, it being well known in the art how to measure a compounds ability to inhibit JAK-3, for example, using standard tests similar to the test described hereinbelow in Example 2 under the heading "Effects of a JAK-3 inhibitor on radiation-induced c-jun activation in DT40 cells."

JAK-3 inhibitors that are useful in the methods of the invention include compounds of formula I:



wherein

X is HN, R<sub>11</sub>N, S, O, CH<sub>2</sub>, or R<sub>11</sub>CH;

R<sub>11</sub> is hydrogen, (C<sub>1</sub>-C<sub>4</sub>)alkyl, or (C<sub>1</sub>-C<sub>4</sub>)alkanoyl;

- 5 R<sub>1</sub>-R<sub>8</sub> are each independently hydrogen, hydroxy, mercapto, amino, nitro, (C<sub>1</sub>-C<sub>4</sub>)alkyl, (C<sub>1</sub>-C<sub>4</sub>)alkoxy, (C<sub>1</sub>-C<sub>4</sub>)alkylthio, or halo; wherein two adjacent groups of R<sub>1</sub>-R<sub>5</sub> together with the phenyl ring to which they are attached may optionally form a fused ring, for example forming a naphthyl or a tetrahydronaphthyl ring; and further wherein the ring formed by the two adjacent groups of R<sub>1</sub>-R<sub>5</sub> may optionally
- 10 be substituted by 1, 2, 3, or 4 hydroxy, mercapto, amino, nitro, (C<sub>1</sub>-C<sub>4</sub>)alkyl, (C<sub>1</sub>-C<sub>4</sub>)alkoxy, (C<sub>1</sub>-C<sub>4</sub>)alkylthio, or halo; and

R<sub>9</sub> and R<sub>10</sub> are each independently hydrogen, (C<sub>1</sub>-C<sub>4</sub>)alkyl, (C<sub>1</sub>-C<sub>4</sub>)alkoxy, halo, or (C<sub>1</sub>-C<sub>4</sub>)alkanoyl; or R<sub>9</sub> and R<sub>10</sub> together are methylenedioxy; or a pharmaceutically acceptable salt thereof.

- 15 The following definitions are used, unless otherwise described: halo is fluoro, chloro, bromo, or iodo. Alkyl, alkanoyl, etc. denote both straight and branched groups; but reference to an individual radical such as "propyl" embraces only the straight chain radical, a branched chain isomer such as "isopropyl" being specifically referred to. (C<sub>1</sub>-C<sub>4</sub>)Alkyl includes methyl, ethyl, propyl, isopropyl,
- 20 butyl, iso-butyl, and sec-butyl; (C<sub>1</sub>-C<sub>4</sub>)alkoxy includes methoxy, ethoxy, propoxy,

isopropoxy, butoxy, iso-butoxy, and sec-butoxy; and (C<sub>1</sub>-C<sub>4</sub>)alkanoyl includes acetyl, propanoyl and butanoyl.

A specific group of compounds are compounds of formula I wherein R<sub>1</sub>-R<sub>5</sub> are each independently hydrogen, mercapto, amino, nitro, (C<sub>1</sub>-C<sub>4</sub>)alkyl, (C<sub>1</sub>-C<sub>4</sub>)alkoxy, (C<sub>1</sub>-C<sub>4</sub>)alkylthio, or halogen.

Another specific group of compounds are compounds of formula I wherein R<sub>9</sub> and R<sub>10</sub> are each independently hydrogen, (C<sub>1</sub>-C<sub>4</sub>)alkyl, halo, or (C<sub>1</sub>-C<sub>4</sub>)alkanoyl; or R<sub>9</sub> and R<sub>10</sub> together are methylenedioxy; or a pharmaceutically acceptable salt thereof.

JAK-3 inhibitors that are useful in the methods of the invention also include compounds of formula I as described in U.S. Patent Application Serial Number 09/087,479 (entitled Quinazolines For Treating Brain Tumor; filed 28 May 1998).

Preferred JAK-3 inhibitors include 4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline and 4-(3'-bromo-4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline, or a pharmaceutically acceptable salt thereof.

Substances that inhibit JAK-3 ("the Substance(s)") can be formulated as pharmaceutical compositions and administered to a mammalian host, such as a human patient in a variety of forms adapted to the chosen route of administration, i.e., orally or parenterally, by intravenous, intramuscular, topical or subcutaneous routes.

Thus, the Substances may be systemically administered, e.g., orally, in combination with a pharmaceutically acceptable vehicle such as an inert diluent or an assimilable edible carrier. They may be enclosed in hard or soft shell gelatin capsules, may be compressed into tablets, or may be incorporated directly with the food of the patient's diet. For oral therapeutic administration, the Substance may be combined with one or more excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of the Substance. The percentage of the compositions and preparations may, of course, be varied and

may conveniently be between about 2 to about 60% of the weight of a given unit dosage form. The amount of Substance in such therapeutically useful compositions is such that an effective dosage level will be obtained.

The tablets, troches, pills, capsules, and the like may also contain the following: binders such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, fructose, lactose or aspartame or a flavoring agent such as peppermint, oil of wintergreen, or cherry flavoring may be added. When the unit dosage form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier, such as a vegetable oil or a polyethylene glycol. Various other materials may be present as coatings or to otherwise modify the physical form of the solid unit dosage form. For instance, tablets, pills, or capsules may be coated with gelatin, wax, shellac or sugar and the like. A syrup or elixir may contain the active compound, sucrose or fructose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring such as cherry or orange flavor. Of course, any material used in preparing any unit dosage form should be pharmaceutically acceptable and substantially non-toxic in the amounts employed. In addition, the Substance may be incorporated into sustained-release preparations and devices.

The Substances may also be administered intravenously or intraperitoneally by infusion or injection. Solutions of the Substance can be prepared in water, optionally mixed with a nontoxic surfactant. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, triacetin, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical dosage forms suitable for injection or infusion can include sterile aqueous solutions or dispersions or sterile powders comprising the Substance which are adapted for the extemporaneous preparation of sterile injectable or infusible solutions or dispersions, optionally encapsulated in liposomes. In all cases, the ultimate dosage form must be sterile, fluid and stable

under the conditions of manufacture and storage. The liquid carrier or vehicle can be a solvent or liquid dispersion medium comprising, for example, water, ethanol, a polyol (for example, glycerol, propylene glycol, liquid polyethylene glycols, and the like), vegetable oils, nontoxic glyceryl esters, and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the formation of liposomes, by the maintenance of the required particle size in the case of dispersions or by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, buffers or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the Substance in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filter sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze drying techniques, which yield a powder of the active ingredient plus any additional desired ingredient present in the previously sterile-filtered solutions.

For topical administration, the Substances may be applied in pure form, i.e., when they are liquids. However, it will generally be desirable to administer them to the skin as compositions or formulations, in combination with a dermatologically acceptable carrier, which may be a solid or a liquid.

Useful solid carriers include finely divided solids such as talc, clay, microcrystalline cellulose, silica, alumina and the like. Useful liquid carriers include water, alcohols or glycols or water-alcohol/glycol blends, in which the Substances can be dissolved or dispersed at effective levels, optionally with the aid of non-toxic surfactants. Adjuvants such as fragrances and additional antimicrobial agents can be added to optimize the properties for a given use. The resultant liquid

compositions can be applied from absorbent pads, used to impregnate bandages and other dressings, or sprayed onto the affected area using pump-type or aerosol sprayers.

Thickeners such as synthetic polymers, fatty acids, fatty acid salts  
5 and esters, fatty alcohols, modified celluloses or modified mineral materials can also be employed with liquid carriers to form spreadable pastes, gels, ointments, soaps, and the like, for application directly to the skin of the user.

Examples of useful dermatological compositions which can be used to deliver the Substances to the skin are known to the art; for example, see Jacquet  
10 et al. (U.S. Pat. No. 4,608,392), Geria (U.S. Pat. No. 4,992,478), Smith et al. (U.S. Pat. No. 4,559,157) and Wortzman (U.S. Pat. No. 4,820,508).

Useful dosages of the compounds of formula I can be determined by comparing their *in vitro* activity, and *in vivo* activity in animal models. Methods for the extrapolation of effective dosages in mice, and other animals, to humans are  
15 known to the art; for example, see U.S. Pat. No. 4,938,949.

Generally, the concentration of the Substance in a liquid composition, such as a lotion, will be from about 0.1-25 wt-%, preferably from about 0.5-10 wt-%. The concentration in a semi-solid or solid composition such as a gel or a powder will be about 0.1-5 wt-%, preferably about 0.5-2.5 wt-%.

20 The amount of the Substance required for use in treatment will vary not only with the particular salt selected but also with the route of administration, the nature of the condition being treated and the age and condition of the patient and will be ultimately at the discretion of the attendant physician or clinician.

In general, however, a suitable dose will be in the range of from  
25 about 0.5 to about 100 mg/kg, e.g., from about 10 to about 75 mg/kg of body weight per day, such as 3 to about 50 mg per kilogram body weight of the recipient per day, preferably in the range of 6 to 90 mg/kg/day, most preferably in the range of 15 to 60 mg/kg/day.

The Substance is conveniently administered in unit dosage form; for example, containing 5 to 1000 mg, conveniently 10 to 750 mg, most conveniently, 50 to 500 mg of active ingredient per unit dosage form.

Ideally, the Substance should be administered to achieve peak plasma  
5 concentrations of from about 0.5 to about 75  $\mu\text{M}$ , preferably, about 1 to 50  $\mu\text{M}$ ,  
most preferably, about 2 to about 30  $\mu\text{M}$ . This may be achieved, for example, by  
the intravenous injection of a 0.05 to 5% solution of the Substance, optionally in  
saline, or orally administered as a bolus containing about 1-100 mg of the  
Substance. Desirable blood levels may be maintained by continuous infusion to  
10 provide about 0.01-5.0 mg/kg/hr or by intermittent infusions containing about 0.4-  
15 mg/kg of the Substance.

The Substance may conveniently be presented in a single dose or as  
divided doses administered at appropriate intervals, for example, as two, three, four  
or more sub-doses per day. The sub-dose itself may be further divided, e.g., into a  
15 number of discrete loosely spaced administrations; such as multiple inhalations from  
an insufflator or by application of a plurality of drops into the eye.

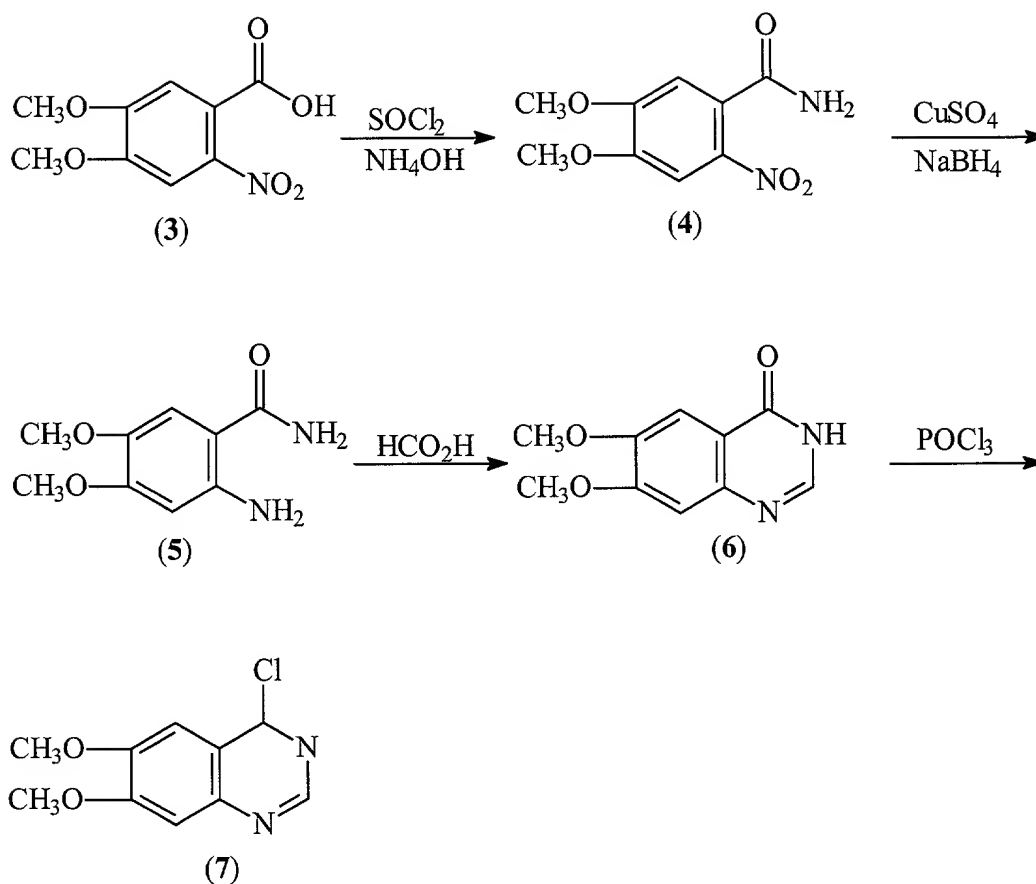
The invention will now be illustrated by the following non-limiting  
Examples.



## Examples

### Example 1. Chemical synthesis and Characterization of JAK-3 Inhibitors

- 5                    Melting points are uncorrected. <sup>1</sup>H NMR spectra were recorded using a Varian Mercury 300 spectrometer in DMSO-d<sub>6</sub> or CDCl<sub>3</sub>. Chemical shifts are reported in parts per million (ppm) with tetramethylsilane (TMS) as an internal standard at zero ppm. Coupling constant (J) are given in hertz and the abbreviations s, d, t, q, and m refer to singlet, doublet, triplet, quartet and multiplet, respectively.
- 10    Infrared spectra were recorded on a Nicolet PROTEGE 460-IR spectrometer. Mass spectroscopy data were recorded on a FINNIGAN MAT 95, VG 7070E-HF G.C. system with an HP 5973 Mass Selection Detector. UV spectra were recorded on BECKMAN DU 7400 and using MeOH as the solvent. TLC was performed on a
- 15    precoated silica gel plate (Silica Gel KGF; Whitman Inc). Silica gel (200-400 mesh, Whitman Inc.) was used for all column chromatography separations. All chemicals were reagent grade and were purchased from Aldrich Chemical Company (Milwaukee, Wis) or Sigma Chemical Company (St. Louis, MO).
- 20                    The common synthetic precursor 4-chloro-6,7-dimethoxyquinazoline (7), used for preparing compounds (1) and (2), was prepared using literature procedures as illustrated in Scheme 1.



**Scheme 1**

4,5-Dimethoxy-2-nitrobenzoic acid (3) was treated with thionyl chloride and then reacted with ammonia to give 4,5-dimethoxy-2-nitrobenzamide (4) as described by F. Nomoto et al. *Chem. Pharm. Bull.* **1990**, 38, 1591-1595. The nitro group in compound (4) was reduced with sodium borohydride in the presence of copper sulfate (see C.L. Thomas *Catalytic Processes and Proven Catalysts* Academic Press, New York (1970)) to give 4,5-dimethoxy-2-aminobenzamide (5) which was cyclized by refluxing with formic acid to give 6,7-dimethoxyquinazoline-4(3H)-one (6). Compound (6) was refluxed with phosphorus oxytrichloride to provide the common synthetic precursor (7).

Compounds **1** and **2** (Figure 4) were prepared from the common synthetic precursor (**7**) and the requisite aniline as follows.

4-(4'-Hydroxyphenyl)-amino-6,7-dimethoxyquinazoline (**1**). A mixture of 448 mg (2 mmol) of 4-chloro-6,7-dimethoxy-quinazoline (**7**) and 2.5 mmol of 4-hydroxyaniline in 20 ml of alcohol (EtOH or MeOH) was refluxed for 8 hours. After cooling triethylamine was added to basify the solution, and the solvent was concentrated to give material that was recrystallized from DMF to give compound (**1**); 84.29%; m.p. 245.0- 248.0 °C; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>): δ 11.21(s, 1H, -NH), 9.70(s, 1H, -OH), 8.74(s, 1H, 2-H), 8.22(s, 1H, 5-H), 7.40(d, 2H, *J* = 8.9 Hz, 2',6'-H), 7.29(s, 1H, 8-H), 6.85(d, 2H, *J* = 8.9 Hz, 3',5'-H), 3.98(s, 3H, -OCH<sub>3</sub>), 3.97(s, 3H, -OCH<sub>3</sub>). UV(MeOH) λ<sub>max</sub>(ε): 203.0, 222.0 , 251.0, 320.0 nm. IR(KBr)u<sub>max</sub>: 3428, 2836, 1635, 1516, 1443, 1234 cm<sup>-1</sup>. GC/MS m/z 298 (M<sup>+</sup>+1, 100.00), 297(M<sup>+</sup>, 26.56), 296( M<sup>+</sup>-1, 12.46).

4-(3'-Bromo-4'-hydroxyphenyl)-amino-6,7-dimethoxy-quinazoline (**2**). A mixture of 448 mg (2 mmol) of 4-chloro-6,7-dimethoxy-quinazoline (**7**) and 2.5 mmol of 3-bromo-4-hydroxyaniline in 20 ml of alcohol (EtOH or MeOH) was refluxed for 8 hours. After cooling, triethylamine was added to basify the solution, and the solvent was concentrated to give material that was recrystallized from DMF to give compound (**2**); 89.90%; m.p. 233.0-233.5 °C; <sup>1</sup>H NMR(DMSO-d<sub>6</sub>): δ 10.08(s, 1H, -NH), 9.38(s, 1H , -OH), 8.40(s, 1H, 2-H), 7.89(d, 1H, *J*<sub>2',5'</sub> = 2.7 Hz, 2'-H), 7.75(s, 1H, 5-H), 7.55(dd, 1H, *J*<sub>5',6'</sub> = 9.0 Hz, *J*<sub>2',6'</sub> = 2.7 Hz, , 6'-H), 7.14(s, 1H, 8-H), 6.97(d, 1H, *J*<sub>5',6'</sub> = 9.0 Hz, 5'-H), 3.92(s, 3H, -OCH<sub>ER</sub>), 3.90(s, 3H, -OCH<sub>3</sub>). UV(MeOH)λ<sub>max</sub>(ε): 203.0, 222.0 , 250.0, 335.0 nm. IR(KBr)u<sub>max</sub>: 3431(br), 2841, 1624, 1498, 1423, 1244 cm<sup>-1</sup>. GC/MS m/z 378( M<sup>+</sup>+2, 90.68), 377(M<sup>+</sup>+1,37.49), 376(M<sup>+</sup>,100.00), 360(M<sup>+</sup>3.63), 298(18.86), 282 (6.65).

## Example 2. Biological Screening

### MATERIALS AND METHODS

#### Cell Lines. The establishment and characterization of

- 5 BTK-deficient, SYK-deficient, and LYN-deficient clones and reconstituted SYK-deficient cell lines of DT-40 chicken lymphoma B-cells were previously reported. The culture medium was RPMI 1640 (Life Technologies; Gaithersburg, MD), supplemented with 1% chicken serum (Sigma; St. Louis, MO), 5% fetal bovine serum (Hyclone, Logan, UT) and 1% penicillin-streptomycin (Life
- 10 Technologies). See Uckun, F. M., Waddick, K. G., Mahajan, S., Jun, X., Takata, M., Bolen, J., and Kurosaki, T. *Science*. 273: 1096-100, 1996; Kurosaki, T. *Curr Opin Immunol*. 9: 309-18, 1997; Kurosaki, T., Johnson, S. A., Pao, L., Sada, K., Yamamura, H., and Cambier, J. C. *J. Exp. Med.* 182: 1815-1823, 1995; and Dibirdik I., Kristupaitis D., Kurosaki T., Tuel-Ahlgren L., Chu A., Pond D., Tuong D., Luben
- 15 R., Uckun F.M. *J. Biol. Chem.* 273(7), pp:4035-4039, 1998.

- Use of PTK Inhibitors.** Cells ( $2 \times 10^6$ /ml) were treated for 24 hours at 37 °C with either (1) the PTK inhibitory isoflavone genistein (Calbiochem, La Jolla, CA) at 111 mM (30 mg/ml) concentration or (2) the Janus family kinase, 3 (JAK-3)-specific PTK inhibitor 4-(3'-bromo-4'-hydroxyphenyl)-amino-6,7-
- 20 dimethoxyquinazoline,  $C_{16}H_{14}Br(N_3O_3)$ , kindly provided by Dr. Xing-Ping Liu, Alexander and Parker Pharmaceutical Inc., Roseville, MN) at 270 mM (100 mg/ml) prior to radiation in order to assess the effects of these agents on radiation-induced *c-jun* activation.

- Irradiation of cells.** Cells ( $2 \times 10^6$  cells/ml) in plastic tissue culture
- 25 flasks were irradiated with 10-20 Gy at a dose rate of 4 Gy/min during log phase growth and under aerobic conditions using a  $^{137}\text{Cs}$  irradiator (J.L. Shephard, Glendale, CA, as previously described by Tuel Ahlgren, L., Jun, X., Waddick, K. G., Jin, J., Bolen, J., and Uckun, F. M. "Role of tyrosine phosphorylation in radiation-induced cell cycle-arrest of leukemic B-cell precursors at the G2-M
- 30 transition checkpoint," *Leuk Lymphoma*. 20: 417-26, 1996; and Uckun, F.M.,

Jaszcz, W., Chandan Langlie, M., Waddick, K.G., Gajl Peczalska, K. and Song, C.W. "Intrinsic radiation resistance of primary clonogenic blasts from children with newly diagnosed B-cell precursor acute lymphoblastic leukemia," *J Clin Invest*. 91:1044-1051, 1993. In some experiments, cells were preincubated with PTK

5 inhibitors for 24 hours prior to irradiation.

***c-jun* probe.** A 506 basepair (bp) *c-jun* probe was obtained by polymerase chain reaction (PCR) amplification of chicken genomic DNA. Primer sequences were determined based upon the sequence of chicken *c-jun* (GenBank accession code CHKJUN). Two primers: 5'-ACTCTGCACC  
10 CAACTACAACGC-3' (SEQ. ID NO: 1) and 5'-CTTCTACCGT  
CAGCTTTACGCG-3' (SEQ ID NO: 2) were used for amplification. Amplification was performed with a mix of Taq polymerase and a proof reading polymerase (eLONGase:Taq polymerase plus *Pyrococcus species* GB-D polymerase, Gibco BRL, Grand Island, NY) on an thermocycler, Ericomp Delta II cycler, using a hot  
15 start. PCR products were subsequently cloned into the cloning vector, PCR 2.1 (Invitrogen, San Diego, CA). An insert of the proper size (506 basepair) was identified as chicken *c-jun* by sequence analysis using PRISM dye terminator cycle sequencing (AmpliTaq® DNA Polymerase, FS) and analyzed on an automated sequencer, ALF express sequencer (Pharmacia Biotech, Piscataway, NJ). A 538  
20 base pair chicken glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe was generated by reverse transcription and subsequent PCR amplification (RT-PCR) from chicken RNA with the following primers:  
5'-AGAGGTGCTGCCCAGAACATCATC-3' (SEQ ID NO: 3) and  
5'-GTGGGGAGACAGAAGGGAACAGA-3' (SEQ ID NO: 4). A 413 bp chicken  
25 B-actin probe was generated by RT-PCR amplification from chicken RNA with the following primers: 5'-GCCCTCTTCCAGCATCTTTCTT-3' (SEQ ID NO: 5) and 5'-TTTATGCGCATTTATGGGT-3' (SEQ ID NO: 6). The amplified cDNAs were cloned into PCR 2.1.

**RNA isolation and Northern blot hybridization analysis.** Total  
30 RNA was extracted from approximately  $2.5 \times 10^7$  cells with Trizol Reagent, a

monophasic solution of phenol and guanidine isothiocyanate as described by Chomczynski, P. and Sacchi, N. "Single-step method of RNA isolation by guanidinium-thiocyanate-phenol-chloroform extraction," *Anal. Biochem.* 162:

156-159, 1987. Poly (A)<sup>+</sup> RNA was isolated directly from 1-3 x 10<sup>8</sup> cells with an  
5 Invitrogen Fast Trak 2.0 mRNA isolation kit. In brief, cells were lysed in a sodium dodecyl sulfate (SDS) lysis buffer containing a proprietary mixture of proteases. The lysate was directly incubated with oligo-dT for absorption and subsequent elution of poly (A)<sup>+</sup> RNA.

Two micrograms of poly (A)<sup>+</sup> or 20 micrograms of total RNA were  
10 denatured in formaldehyde/formamide loading dye at 65° prior to loading onto a 1% agarose-formaldehyde denaturing gel. Transcript sizes were determined relative to RNA markers of 0.5-9 kb. The gels were stained with Radiant Red in H<sub>2</sub>O to check loading and integrity of RNA prior to transfer. The RNA was subsequently transferred to positively charged nylon membrane with 20X standard sodium  
15 citrate(SSC) transfer buffer (1XSSC= 0.15 M sodium chloride-0.015 M sodium citrate) by downward capillary transfer. The *c-jun* fragment was radiolabeled by random priming with [(-<sup>32</sup>P)-dCTP (3000 Ci/mM) [Amersham, Arlington Heights, IL] (40). Northern blots were hybridized overnight at 42 °C in prehybridization/hybridization solution (50% formamide with proprietary blocking  
20 and background reduction reagents; Ambion, Austin, TX) for 16-24 hours and unbound probe was removed by washing to a final stringency of 0.1% SDS, 0.1XSSC (65 °C). The blots were analyzed both by autoradiography and using the BioRad Storage Phosphor Imager System (BioRad, Hercules, CA) for quantitative scanning. The blots were subsequently stripped in boiling 0.1% SDS, and then  
25 rehybridized with a chicken GAPDH and/or chicken (β-actin probe to normalize for loading differences.

## RESULTS AND DISCUSSION

**Exposure of DT-40 chicken lymphoma B-cells to ionizing  
30 radiation activates the *c-jun* protooncogene.** Exposure of human lymphoma

B-cells to 10-20 Gy-rays results in enhanced *c-jun* expression with a maximum response at 1-2 hours (Chae, H. P., Jarvis, L. J., and Uckun, F. M. *Cancer Res.* 53: 447-51, 1993). It has also been reported that ionizing radiation triggers in DT-40 chicken lymphoma B-cells biochemical and biological signals similar to those in human lymphoma B-cells (Uckun, F. M., Waddick, K. G., Mahajan, S., Jun, X., Takata, M., Bolen, J., and Kurosaki, T. *Science*. 273: 1096-100, 1996). In order to determine if DT-40 chicken lymphoma B-cells show a similar *c-jun* response to ionizing radiation, DT-40 cells were irradiated with 5, 10, 15 or 20 Gy and examined total RNA harvested from cells 2 or 4 hours after radiation exposure for expression levels of 1.8 kb chicken *c-jun* transcripts by quantitative Northern blot analysis. As shown in Figure 1A, radiation exposure increased the level of *c-jun* transcripts in a dose-and time-dependent manner without significantly affecting the GAPDH transcript levels with a maximum stimulation index (SI) [as determined by comparison of the *c-jun*/GAPDH ratios in non-irradiated versus irradiated cells] of 3.1, 4 hours after 20 Gy. In seven additional independent experiments, the stimulation index for 20 Gy ionizing radiation at 2 hours after radiation exposure ranged from 2.4 to 3.8 (mean (SE) =  $2.9 \pm 0.4$ ).

The role of PTK in radiation-induced activation of *c-jun* expression in chicken lymphoma B cells was examined next, since PTK inhibitors were shown to prevent radiation-induced *c-jun* activation in human lymphoma B-cells. As shown in Figure 1B, ionizing radiation did not significantly enhance *c-jun* expression levels in DT-40 cells treated with the PTK-inhibitory isoflavone, genistein (stimulation index=1.1) indicating that activation of a PTK is required for radiation-induced *c-jun* expression in chicken lymphoma B cells as well. These findings established DT-40 chicken lymphoma B-cells as a suitable model to further elucidate the molecular mechanism of radiation-induced *c-jun* activation.

**Cytoplasmic protein tyrosine kinases BTK, LYN, and SYK are not required for radiation induced *c-jun* activation.** BTK is abundantly expressed in lymphoma B-cells and its activation has been shown to be required for radiation-induced apoptosis of DT-40 cells (Uckun, F. M., Waddick, K. G.,

Mahajan, S., Jun, X., Takata, M., Bolen, J., and Kurosaki, T. *Science*. 273:

1096-100, 1996). DT-40 cells rendered BTK-deficient by targeted disruption of the BTK genes do not undergo apoptosis after radiation exposure. Therefore, we set out to determine if BTK could be the PTK responsible for radiation-induced *c-jun*

5 activation as well, by comparing the levels of *c-jun* induction in BTK-deficient (BTK<sup>-</sup>) versus wild-type DT-40 cells. Contrary to our expectations, 20 Gy ionizing radiation did not fail to induce *c-jun* expression in BTK-deficient DT-40 cells in any of the three independent experiments performed. The stimulation indices ranged from 1.6 to 3.9 (mean  $\pm$  SE =  $2.4 \pm 0.5$ ) (Figure 2). Thus, ionizing  
10 radiation-induced increases in *c-jun* transcript levels do not depend upon the presence of BTK.

Since SYK is also abundantly expressed in DT-40 cells and is rapidly activated after ionizing radiation, we next examined if SYK might be the PTK responsible for radiation-induced increases in *c-jun* transcript levels. As shown in  
15 Figure 3A, 20 Gy ionizing radiation enhanced *c-jun* expression in SYK<sup>-</sup> DT-40 cells rendered SYK-deficient by targeted gene disruption even though the stimulation indices observed in five independent experiments were lower than from those in wild-type cells ( $1.9 \pm 0.2$ , vs  $2.9 \pm 0.4$ ,  $p < 0.01$ ). Thus, SYK is not required for radiation-induced *c-jun* activation in DT-40 cells but it may participate in  
20 generation of an optimal signal.

DT-40 cells express high levels of LYN but do not express other members of the Src PTK family, including BLK, HCK, SRC, FYN, or YES at detectable levels (see Uckun, F. M., Waddick, K. G., Mahajan, S., Jun, X., Takata, M., Bolen, J., and Kurosaki, T. *Science*. 273: 1096-100, 1996; Kurosaki, T.,  
25 Johnson, S. A., Pao, L., Sada, K., Yamamura, H., and Cambier, J. C. "Role of the Syk autophosphorylation site and SH2 domains in B cell antigen receptor signaling," *J. Exp. Med.* 182: 1815-1823, 1995; and Takata, M., Homma, Y., and Kurosaki, T. "Requirement of phospholipase C- $\gamma$ 2 activation in surface immunoglobulin M-induced B cell apoptosis," *J Exp Med.* 182: 907-914, 1995.  
30 Since it has previously been demonstrated that SRC family PTK are essential for



UV-stimulated increases in *c-jun* expression, we postulated that the predominant SRC-family member, LYN, might mediate radiation-induced *c-jun* expression in DT-40 cells. To test this hypothesis, we examined the ability of ionizing radiation to activate *c-jun* expression in DT-40 cells rendered LYN-deficient by targeted gene  
 5 disruption. LYN-deficient (LYN<sup>-</sup>) cells showed enhanced *c-jun* expression after irradiation, however the stimulation indices were lower than those in wild-type DT-40 (Figure 3B). Since LYN and SYK have been shown to cooperate in the generation of other signals in B-cells (see Kurosaki, T. "Molecular mechanisms in B cell antigen receptor signaling," *Curr Opin Immunol.* 9: 309-18, 1997), the ability of  
 10 ionizing radiation to induce *c-jun* expression in LYN<sup>-</sup>SYK<sup>-</sup> DT-40 cells, generated by targeted disruption of the *syk* gene in LYN<sup>-</sup> deficient DT-40 cells was examined. As shown in Figure 3B, LYN<sup>-</sup>SYK<sup>-</sup> DT-40 cells showed elevated *c-jun* transcript levels after irradiation, indicating that the *c-jun* response does not depend on either of these PTK, either alone or in cooperation. Similar to SYK, LYN is not required  
 15 for radiation-induced *c-jun* activation in DT-40 cells but it may participate in generation of an optimal response.

Interestingly, in four independent experiments, we observed higher baseline expression levels of *c-jun* in SYK<sup>-</sup> DT-40 cells than in wild-type DT-40 cells (Range: 1.4 - 2.3-fold, mean  $\pm$  SE = 1.6  $\pm$  0.2-fold), suggesting that Syk may  
 20 be involved in regulation of baseline *c-jun* levels. To further explore this possibility, we compared *c-jun* levels in SYK<sup>-</sup> cells to those of SYK<sup>-</sup> cells reconstituted with wild-type or kinase domain mutant (K<sup>-</sup>) *syk* gene. We observed that reconstitution with wild-type *syk* reduced the higher baseline expression levels of *c-jun* in SYK<sup>-</sup> cells, whereas reconstitution with a K<sup>-</sup> *syk* failed to reduce *c-jun*  
 25 levels (data not shown). These results implicate SYK as a negative regulator of *c-jun* expression. This novel function of SYK seems to depend on its kinase domain.

**Effects of a JAK-3 inhibitor on radiation-induced c-jun activation in DT40-cells.** B-cell signal transduction events direct fundamental  
 30 decisions regarding cell survival during periods of oxidative stress. A better

understanding of the dynamic interplay between B-cell signaling pathways is needed to determine how vital decisions are dictated during intracellular oxidation changes. STAT proteins (signal transducers and activators of transcription) are a family of DNA binding proteins that were identified during a search for interferon (IFN)  $\alpha$ - or g-stimulated gene transcription targets. There are presently seven STAT family members. The JAK family of cytoplasmic protein kinases were originally demonstrated to also function in IFN signaling, and are now known to participate in a broad range of receptor-activated signal cascades. Different ligands and cell activators employ specific JAK and STAT family members. The basic model for STAT activation suggests that in unstimulated cells, latent forms of STATs are predominantly localized within the cytoplasm. Ligand binding induces STAT proteins to associate with intracellular phosphotyrosine residues of transmembrane receptors. Once STATs are bound to receptors, receptor-associated JAK kinases phosphorylate the STAT proteins. STAT proteins then dimerize through specific reciprocal SH2-phosphotyrosine interactions and may form complexes with other DNA-binding proteins. STAT complexes translocate to the nucleus and interact with DNA response elements to enhance transcription of target genes. Signaling events regulating apoptotic responses have been shown to utilize STAT proteins. Notably, a recent study demonstrated JAK activation by tyrosine phosphorylation in cells that are exposed to reactive oxygen intermediates, which in-turn lead to tyrosine phosphorylation and activation of STAT-1, STAT-3 and STAT-6.

After establishing that LYN, BTK, and SYK kinases are not required for radiation-induced c-jun activation, we set out to determine if c-jun activation is functionally linked to the JAK-STAT pathway. To this end, we examined the effects of a JAK-3 inhibitory novel quinazoline derivative on c-jun expression levels in irradiated DT-40 cells. To identify a potent JAK-3 specific inhibitor, the effects of two novel quinazoline derivatives on the enzymatic activity of JAK-1, JAK-2, and JAK-3 were examined using Sf21 cells that were infected with baculovirus expression vectors for these kinases, using standard methods (Figure 4). Infected cells were harvested, JAKs were immunoprecipitated with appropriate antibodies

(anti-JAK-1: (HR-785), cat# sc-277, rabbit polyclonal IgG affinity purified, 0.1 mg/ml, Santa Cruz Biotechnology; anti-JAK-2: (C-20)-G, cat # sc-294-G, goat polyclonal IgG affinity purified, 0.2 mg/ml, Santa Cruz Biotechnology; anti-JAK-3: (C-21), cat # sc-513, rabbit polyclonal IgG affinity purified, 0.2 mg/ml, Santa Cruz Biotechnology), and kinase assays were performed following a 1 hour exposure of the immunoprecipitated Jaks to the quinazoline compounds, as described by Uckun, F. M., Waddick, K. G., Mahajan, S., Jun, X., Takata, M., Bolen, J., and Kurosaki, T. *Science*. 273: 1096-100, **1996**; Uckun F.M, Evans W.E, Forsyth C.J, Waddick K.G, T-Ahlgren L., Chelstrom L.M, Burkhardt A., Bolen J., Myers D.E. *Science* 267:886-891, **1995**; and Myers D.E., Jun X., Waddick K.G., Forsyth C., Chelstrom L.M., Gunther R.L., Tumer N.E, Bolen J., Uckun F.M. *Proc Nat'l Acad Sci USA* 92: 9575-9579, **1995**; and Tuel Ahlgren, L., Jun, X., Waddick, K. G., Jin, J., Bolen, J., and Uckun, F. M. *Leuk Lymphoma*. 20: 417-26, **1996**.

As shown in Figure 4B, both compounds inhibited JAK-3 (Figures B.3 and B.4) but not JAK-1 (Figure B.1) or JAK-2 (Figure B.2) (Figure 4D). Electrophoretic Mobility Shift Assays (EMSAs) were performed to examine the effects of both compounds on cytokine-induced STAT activation. Specifically, 32Dc11/IL2R $\beta$  cells (gift from James Ihle, St. Jude Children's Research Hospital) were exposed at  $8 \times 10^6$ /ml in RPMI supplemented with FBS to the JAK-3 inhibitors at a final concentration of 10  $\mu$ g/ml in 1% DMSO for 1 hour and subsequently stimulated with IL2 or IL3 as indicated. Cells were collected after 15 minutes and resuspended in lysis buffer (100 mM Tris-HCl pH 8.0, 0.5% NP-40, 10% glycerol, 100 mM EDTA, 0.1 mM NaVO<sub>3</sub>, 50 mM NaF, 150 mM NaCl, 1 mM DTT, 3 (g/ml Aprotinin, 2 g/ml Pepstatin A, 1 (g/ml Leupeptin and 0.2 mM PMSF). Lysates were precleared by centrifugation for 30 minutes. Cell extracts (approximately 10 g) were incubated with 2  $\mu$ g of poly(dI-dC) for 30 minutes, followed by a 30 minute incubation with 1 ng of poly nucleotide kinase-<sup>32</sup>P labeled double stranded DNA oligonucleotide representing the IRF-1 STAT DNA binding sequence (Santa Cruz Biotechnology, Santa Cruz, CA). Samples were resolved by nondenaturing PAGE and visualized by autoradiography. As shown in Figure 4C,

both compounds inhibited the JAK-3-dependent STAT activation after stimulation with IL-2, but they did not affect the JAK-1/JAK-2-dependent STAT activation after stimulation with IL-3. Compound 2 was selected for further experiments designed to examine the effects of JAK-3 inhibition on radiation-induced c-jun activation.

5           As shown in Figure 5, ionizing radiation failed to induce c-jun expression in DT-40 cells treated with the JAK-3 inhibitor. This demonstrates that JAK-3 inhibitors are capable of inhibiting radiation induced c-jun expression.

          In untreated cells, c-jun expression is induced by exposure to DNA-damaging chemical agents and by exposure to radiation. Thus, c-jun expression is  
10       an early marker of cellular response to such DNA-damaging agents. It has been shown that compounds that inhibit JAK-3 are capable of inhibiting the expression of c-jun. Accordingly, JAK-3 inhibitors may be useful to prevent or treat diseases or conditions that result from exposure to DNA-damaging agents.

          JAK-3 maps to human chromosome 19p12-13.1. A cluster of genes  
15       encoding protooncogenes and transcription factors is also located near this region. JAK-3 expression has been demonstrated in mature B-cells as well as B-cell precursors. JAK-3 has also been detected in leukemic B-cell precursors and lymphoma B-cells. The physiological roles for JAK-3 have been borne out through targeted gene disruption studies in mice, the genetic analysis of patients with severe  
20       combined immunodeficiency, and biochemical studies of JAK-3 in cell lines. A wide range of stimuli result in JAK-3 activation in B-cells, including interleukin 7 and interleukin 4. The B-cell marker CD40 constitutively associates with JAK-3 and ligation of CD40 results in JAK-3 activation which has been shown to be mandatory for CD40-mediated gene expression. Constitutive activity of JAK-3 has  
25       been observed in v-abl transformed pre-B cells and coimmunoprecipitations show that v-abl physically associates with JAK-3 implicating JAK-3 in v-abl induced cellular transformation. See Ihle, J. N. "Janus kinases in cytokine signalling," *Philos Trans R Soc Lond B Biol Sci* 351:159-66, 1996; Leonard, W. J. "STATs and cytokine specificity," *Nat Med* 2:968-9, 1996; Levy, D. E. "The house that Jak/Stat  
30       built," *Cytokine Growth Factor Rev* 8:81-90, 1997; Riedy, M.C. et al. "Genomic

- sequence, organization, and chromosomal localization of human JAK-3," *Genomics* 37, 57-61, **1996**; Safford, M.G., Levenstein, M., Tsifrina, E., Amin, S., Hawkins, A.L., Griffin, C.A., Civin, C.I. and Small, D. "JAK-3: expression and mapping to chromosome 19p12-13.1" [published erratum appears in *Exp Hematol* 1997 Jul;25(7):650]. *Exp Hematol* 25, 374-86, **1997**; Kumar, A., Toscani, A., Rane, S. and Reddy, E.P. "Structural organization and chromosomal mapping of JAK-3 locus," *Oncogene* 13, 2009-14, **1996**; Hoffman, S.M., Lai, K.S., Tomfohrde, J., Bowcock, A., Gordon, L.A. and Mohrenweiser, H.W. "JAK-3 maps to human chromosome 19p12 within a cluster of proto-oncogenes and transcription factors," *Genomics* 43, 109-111, **1997**; Tortolani, P.J. et al. "Regulation of JAK-3 expression and activation in human B cells and B cell malignancies," *J Immunol* 155, 5220-6, **1995**; Sharfe, N., Dadi, H.K., JJ, O.S. and Roifman, C.M. "JAK-3 activation in human lymphocyte precursor cells," *Clin Exp Immunol* 108, 552-6, **1997**; Gurniak, C.B. and Berg, L.J. "Murine JAK-3 is preferentially expressed in hematopoietic tissues and lymphocyte precursor cells," *Blood* 87, 3151-60, **1996**; Rolling, C., Treton, D., Beckmann, P., Galanaud, P. and Richard, Y. "JAK-3 associates with the human interleukin 4 receptor and is tyrosine phosphorylated following receptor triggering," *Oncogene* 10, 1757-61, **1995**; Rolling, C., Treton, D., Pellegrini, S., Galanaud, P. and Richard, Y. "IL4 and IL13 receptors share the gamma c chain and activate STAT6, STAT3 and STAT5 proteins in normal human B cells," *FEBS Lett* 393, 53-6, **1996**; Hanissian, S.H. and Geha, R.S. "JAK-3 is associated with CD40 and is critical for CD40 induction of gene expression in B cells," *Immunity* 6, 379-87, **1997**; Danial, N.N., Pernis, A. and Rothman, P.B. "Jak-STAT signaling induced by the v-abl oncogene," *Science* 269, 1875-7, **1995**.

25

## Summary

Exposure of B-lineage lymphoid cells to ionizing radiation induces an elevation of *c-jun* protooncogene mRNA levels. This signal is abrogated by protein tyrosine kinase (PTK) inhibitors, indicating that activation of an as yet unidentified PTK is mandatory for radiation-induced *c-jun* expression.

30

Experimental evidence shows that the cytoplasmic tyrosine kinases BTK, SYK and LYN are not required for this signal. Lymphoma B-cells rendered deficient for LYN, SYK or both by targeted gene disruption showed increased *c-jun* expression levels after radiation exposure, but the magnitude of the stimulation was lower than in wild-type cells. Thus, these PTK may participate in the generation of an optimal signal. Notably, inhibitors of Janus family kinase 3 (JAK-3) abrogated radiation-induced *c-jun* activation. This suggests that JAKs are important regulators of radiation-induced *c-jun* activation, and that JAK-3 inhibitors are useful for preventing or treating diseases or conditions that result from chemical-induced or radiation-induced *c-jun* activation.

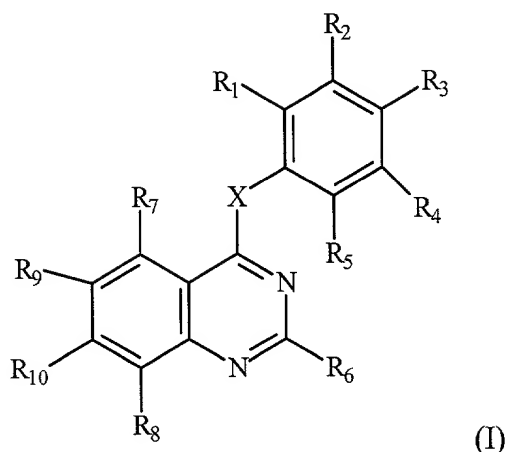
All publications, patents, and patent documents are incorporated by reference herein, as though individually incorporated by reference. The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

## CLAIMS

What is claimed is:

1. A method comprising inhibiting c-jun activation in mammalian or avian cells by contacting the cells with a substance that inhibits the activity of Janus family kinase 3 (JAK-3).
2. The method of claim 1 wherein the c-jun activation results from exposure of the cells to ara-C, a topoisomerase II inhibitor, ultraviolet radiation, an alkylating agent, or ionizing radiation.
3. The method of claim 1 wherein the c-jun activation results from exposure of the cells to ultraviolet radiation or ionizing radiation.
4. The method of claim 1 wherein the contacting is performed *in vitro*.
5. The method of claim 1 wherein the contacting is performed *in vitro*.
6. The method of claim 2 wherein the contacting occurs prior to the exposure.
7. The method of claim 2 wherein the contacting occurs after the exposure.
8. The method of claim 1 wherein the substance is a protein.

9. The method of claim 1 wherein the substance is a compound of formula I:



wherein

X is HN, R<sub>11</sub>N, S, O, CH<sub>2</sub>, or R<sub>11</sub>CH;

R<sub>11</sub> is hydrogen, (C<sub>1</sub>-C<sub>4</sub>)alkyl, or (C<sub>1</sub>-C<sub>4</sub>)alkanoyl;

R<sub>1</sub>-R<sub>8</sub> are each independently hydrogen, hydroxy, mercapto, amino, nitro, (C<sub>1</sub>-C<sub>4</sub>)alkyl, (C<sub>1</sub>-C<sub>4</sub>)alkoxy, (C<sub>1</sub>-C<sub>4</sub>)alkylthio, or halo; wherein two adjacent groups of R<sub>1</sub>-R<sub>5</sub> together with the phenyl ring to which they are attached may optionally form a fused ring, for example forming a naphthyl or a tetrahydronaphthyl ring; and further wherein the ring formed by the two adjacent groups of R<sub>1</sub>-R<sub>5</sub> may optionally be substituted by 1, 2, 3, or 4 hydroxy, mercapto, amino, nitro, (C<sub>1</sub>-C<sub>4</sub>)alkyl, (C<sub>1</sub>-C<sub>4</sub>)alkoxy, (C<sub>1</sub>-C<sub>4</sub>)alkylthio, or halo; and

R<sub>9</sub> and R<sub>10</sub> are each independently hydrogen, (C<sub>1</sub>-C<sub>4</sub>)alkyl, (C<sub>1</sub>-C<sub>4</sub>)alkoxy, halo, or (C<sub>1</sub>-C<sub>4</sub>)alkanoyl; or R<sub>9</sub> and R<sub>10</sub> together are methylenedioxy; or a pharmaceutically acceptable salt thereof.

10. The method of claim 1 wherein the substance is 4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline or 4-(3'-bromo-4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline; or a pharmaceutically acceptable salt thereof.

11. The method of claim 1 wherein the cells are mammalian.

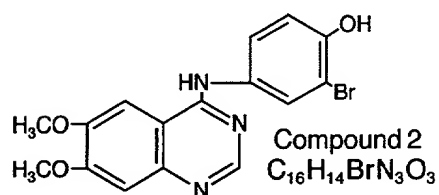
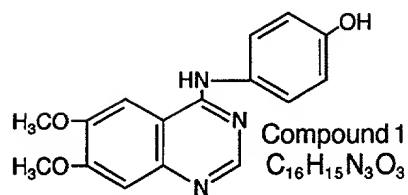


12. The method of claim 1 wherein the cells are human.
13. The method of claim 1 wherein the cells are avian.
14. A therapeutic method for preventing or treating a pathological condition in a mammal wherein c-jun activation is implicated and inhibition of its activation is desired comprising administering to a mammal in need of such therapy, an effective amount of a substance that inhibits the activity of JAK-3.

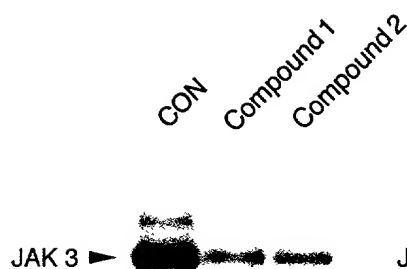
### Abstract

The invention provides a method for inhibiting c-jun activation in mammalian or avian cells comprising contacting the cells with a substance that inhibits the activity of Janus family kinase 3 (JAK-3). The invention also provides a therapeutic method for preventing or treating a pathological condition in a mammal wherein c-jun activation is implicated and inhibition of its activation is desired comprising administering to a mammal in need of such therapy, an effective amount of a substance that inhibits the activity of JAK-3. Novel compounds that are JAK-3 inhibitors, as well as pharmaceutical compositions comprising the compounds are also provided.

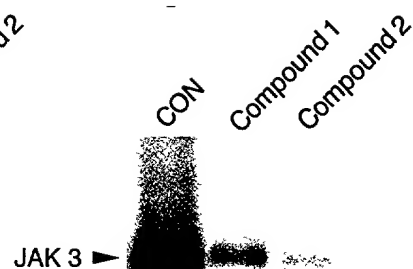
**"Express Mail" mailing label number:** EL273421044 US  
**Date of Deposit:** JUNE 30, 1999  
I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231  
**Printed Name:** Chris Hammond  
**Signature:** Chris Hammond



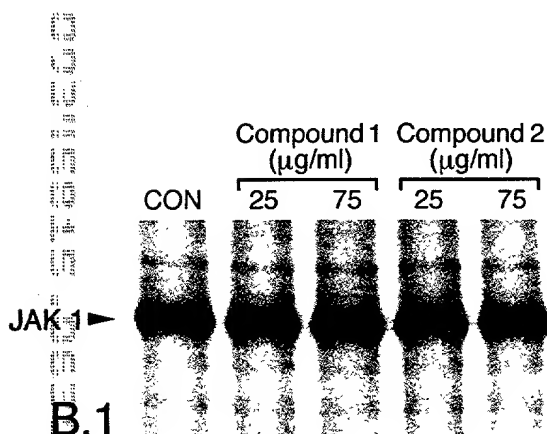
A.



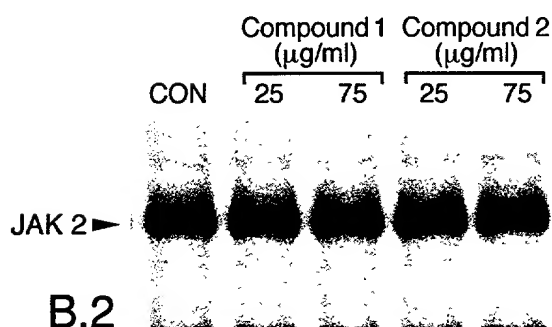
B.3



B.4



B.1



B.2

Compound 1	-	-	-	+	+	+	-	-	-
Compound 2	-	-	-	-	-	-	+	+	+
IL-2	-	+	-	-	+	-	-	+	-
IL-3	-	-	+	-	-	+	-	-	+
Lane	1	2	3	4	5	6	7	8	9

C.

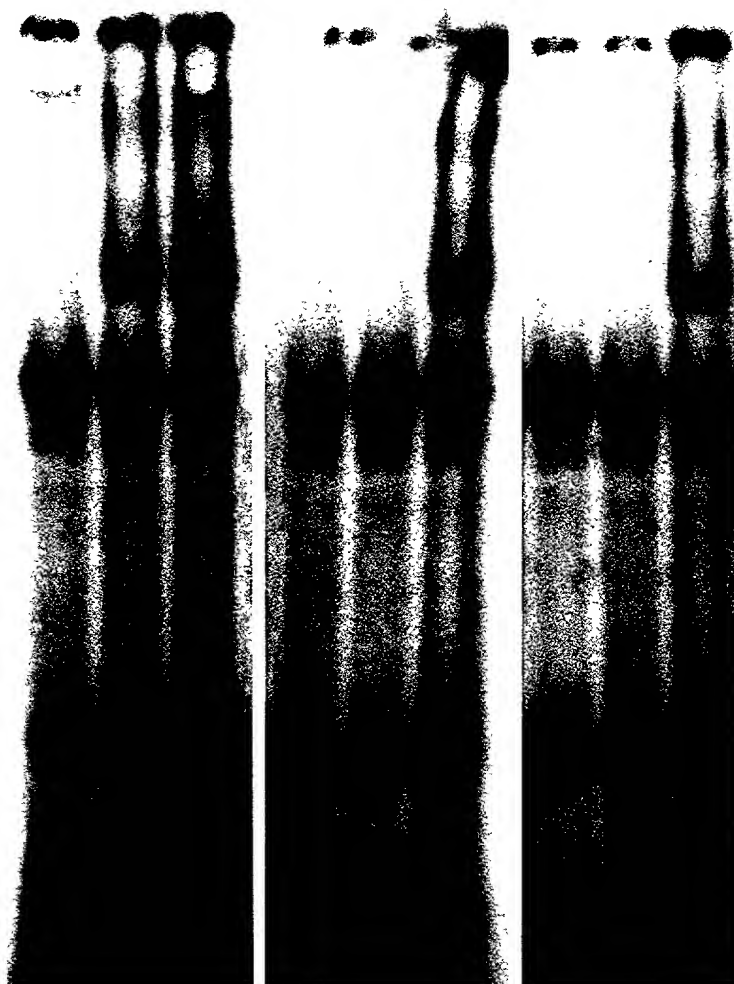


Figure 1

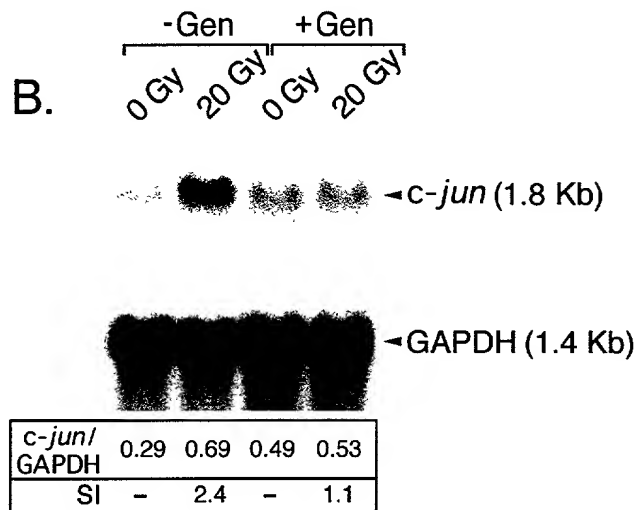
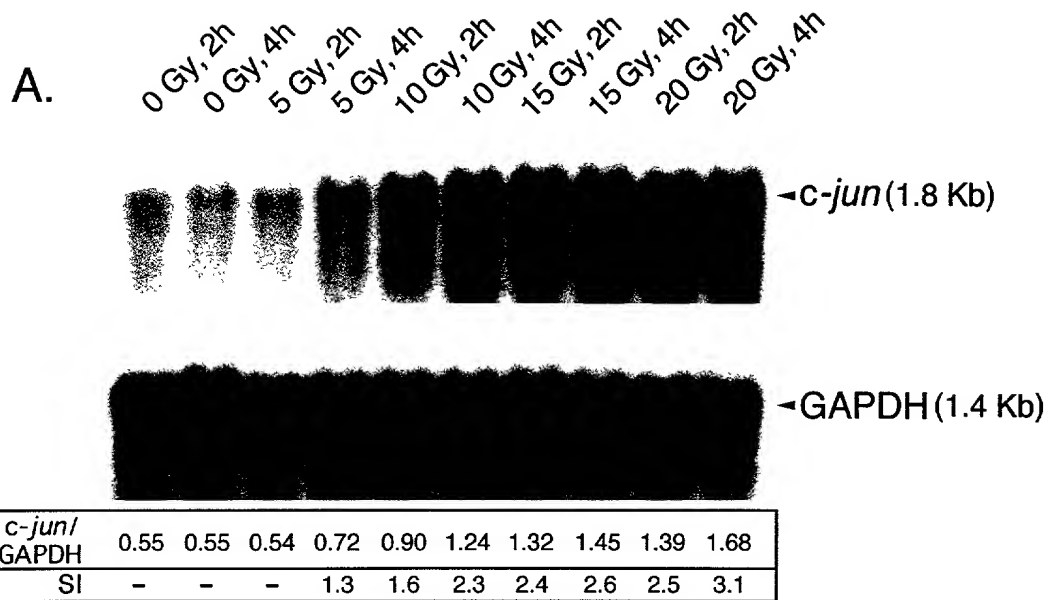


Figure 2

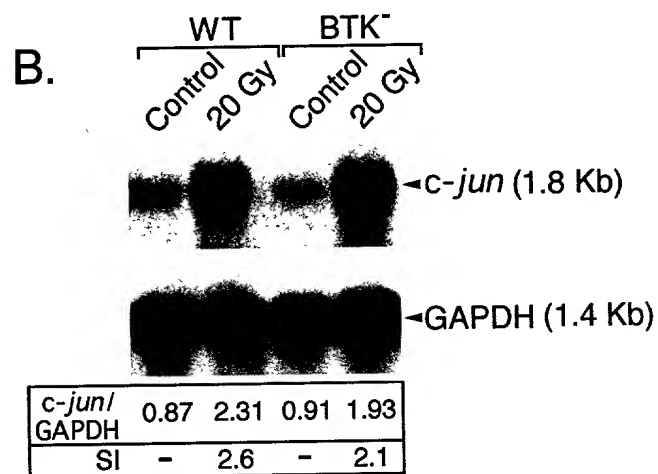
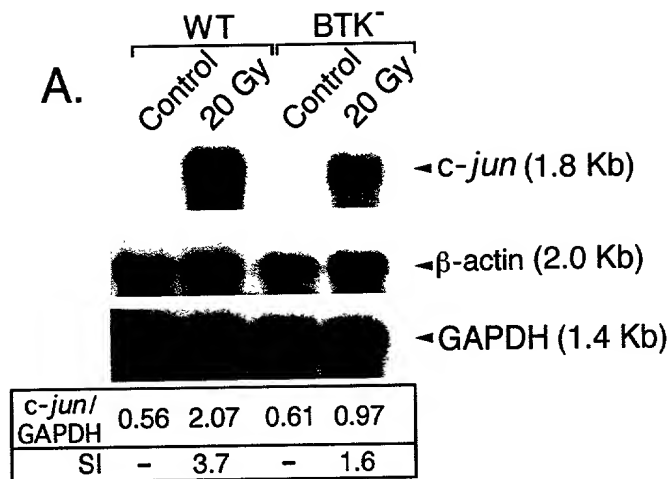


Figure 3

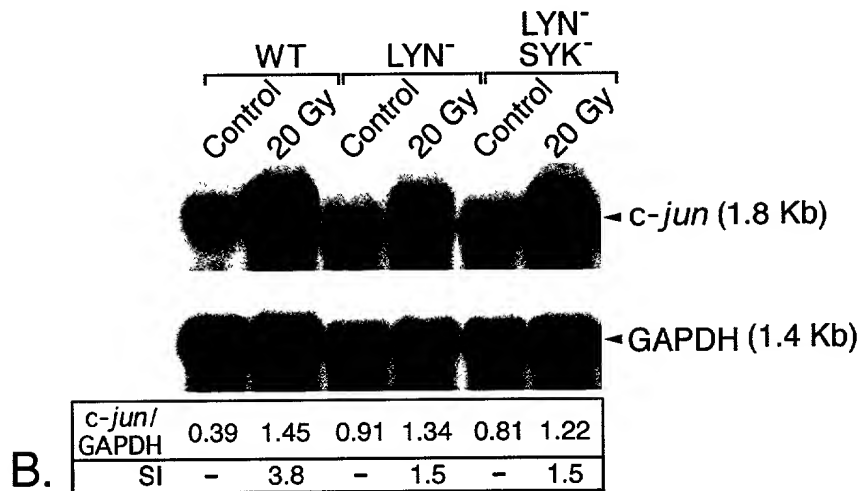
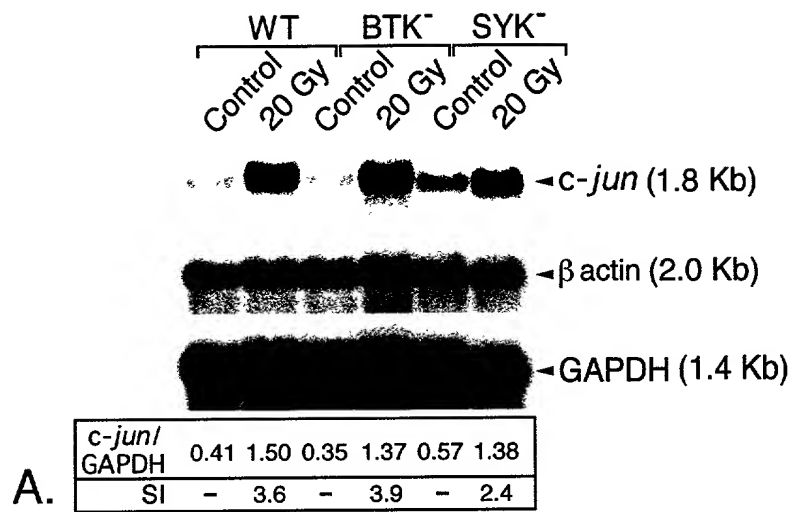


Figure 4

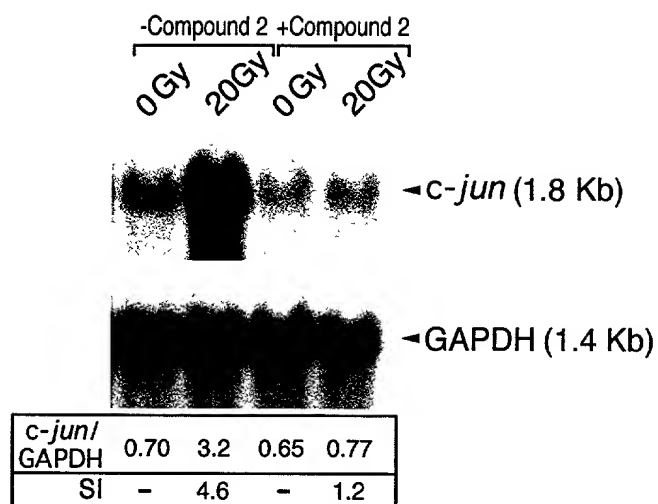


Figure 5

SCHWEGMAN, LUNDBERG, WOESSNER &amp; KLUTH, P.A.

# United States Patent Application

## COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; that

I verily believe I am the original, first and sole inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled: **METHOD FOR INHIBITING C-JUN EXPRESSION USING JAK-3 INHIBITORS.**

The specification of which is attached hereto.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with 37 C.F.R. § 1.56 (attached hereto). I also acknowledge my duty to disclose all information known to be material to patentability which became available between a filing date of a prior application and the national or PCT international filing date in the event this is a Continuation-In-Part application in accordance with 37 C.F.R. § 1.63(e).

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on the basis of which priority is claimed:

**No such claim for priority is being made at this time.**

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

**Application Number**  
**60/091,150**

**Filing Date**  
**June 30, 1998**

I hereby claim the benefit under 35 U.S.C. § 120 or 365(c) of any United States and PCT international application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose material information as defined in 37 C.F.R. § 1.56(a) which became available between the filing date of the prior application and the national or PCT international filing date of this application:

**No such claim for priority is being made at this time.**



I hereby appoint the following attorney(s) and/or patent agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith:

Adams, Gregory J.	Reg. No. P-44,494	Forrest, Bradley A.	Reg. No. 30,837	Mates, Robert E.	Reg. No. 35,271
Adams, Matthew W.	Reg. No. 43,459	Harris, Robert J.	Reg. No. 37,346	McCrackin, Ann M.	Reg. No. 42,858
Anglin, J. Michael	Reg. No. 24,916	Holloway, Sheryl S.	Reg. No. 37,850	Nama, Kash	Reg. No. 44,255
Arora, Suneel	Reg. No. 42,267	Huebsch, Joseph C.	Reg. No. 42,673	Nielsen, Walter W.	Reg. No. 25,539
Bianchi, Timothy E.	Reg. No. 39,610	Jurkovich, Patti J.	Reg. No. P-44,813	Oh, Allen J.	Reg. No. 42,047
Billion, Richard E.	Reg. No. 32,836	Kalis, Janal M.	Reg. No. 37,650	Padys, Danny J.	Reg. No. 35,635
Black, David W.	Reg. No. 42,331	Kaufmann, John D.	Reg. No. 24,017	Parker, J. Kevin	Reg. No. 33,024
Brennan, Thomas F.	Reg. No. 35,075	Klima-Silberg, Catherine I.	Reg. No. 40,052	Peacock, Gregg A.	Reg. No. P-45,001
Brooks, Edward J., III	Reg. No. 40,925	Kluth, Daniel J.	Reg. No. 32,146	Polglaze, Daniel J.	Reg. No. 39,801
Chu, Dinh C.P.	Reg. No. P-41,676	Lacy, Rodney L.	Reg. No. 41,136	Schwegman, Micheal L.	Reg. No. 25,816
Clark, Barbara J.	Reg. No. 38,107	Leffert, Thomas W.	Reg. No. 40,697	Sieffert, Kent J.	Reg. No. 41,312
Dahl, John M.	Reg. No. P-44,639	Lemaire, Charles A.	Reg. No. 36,198	Slifer, Russell D.	Reg. No. 39,838
Drake, Eduardo E.	Reg. No. 40,594	Litman, Mark A.	Reg. No. 26,390	Steffey, Charles E.	Reg. No. 25,179
Eliseeva, Maria M.	Reg. No. 43,328	Lundberg, Steven W.	Reg. No. 30,568	Terry, Kathleen R.	Reg. No. 31,884
Embretson, Janet E.	Reg. No. 39,665	Mack, Lisa K.	Reg. No. 42,825	Viksnins, Ann S.	Reg. No. 37,748
Fogg, David N.	Reg. No. 35,138	Maki, Peter C.	Reg. No. 42,832	Woessner, Warren D.	Reg. No. 30,440
Fordebacher, Paul J.	Reg. No. 42,546	Malen, Peter L.	Reg. No. P-44,894		

I hereby authorize them to act and rely on instructions from and communicate directly with the person/assignee/attorney/firm/organization/who/which first sends/sent this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instruct Schwegman, Lundberg, Woessner & Kluth, P.A. to the contrary.

Please direct all correspondence in this case to **Schwegman, Lundberg, Woessner & Kluth, P.A.** at the address indicated below:

**P.O. Box 2938, Minneapolis, MN 55402**

**Telephone No. (612)373-6900**

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full Name of sole inventor : **Fatih M. Uckun**  
Citizenship: **Turkey**  
Post Office Address: **12590 Ethan Avenue North  
White Bear Lake, MN 55110**

Residence: **White Bear Lake, MN**

Signature: \_\_\_\_\_ Date: \_\_\_\_\_  
**Fatih M. Uckun**

Full Name of inventor:  
Citizenship:  
Post Office Address:

Residence:

Signature: \_\_\_\_\_ Date: \_\_\_\_\_

§ 1.56 Duty to disclose information material to patentability.

(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is canceled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is canceled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in a patent was cited by the Office or submitted to the Office in the manner prescribed by §§ 1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

- (1) prior art cited in search reports of a foreign patent office in a counterpart application, and
- (2) the closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.

(b) Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

- (1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or
- (2) It refutes, or is inconsistent with, a position the applicant takes in:
  - (i) Opposing an argument of unpatentability relied on by the Office, or
  - (ii) Asserting an argument of patentability.

A prima facie case of unpatentability is established when the information compels a conclusion that a claim is unpatentable under the preponderance of evidence, burden-of-proof standard, giving each term in the claim its broadest reasonable construction consistent with the specification, and before any consideration is given to evidence which may be submitted in an attempt to establish a contrary conclusion of patentability.

(c) Individuals associated with the filing or prosecution of a patent application within the meaning of this section are:

- (1) Each inventor named in the application;
- (2) Each attorney or agent who prepares or prosecutes the application; and
- (3) Every other person who is substantively involved in the preparation or prosecution of the application and who is associated with the inventor, with the assignee or with anyone to whom there is an obligation to assign the application.

(d) Individuals other than the attorney, agent or inventor may comply with this section by disclosing information to the attorney, agent, or inventor.